### References and Notes

1. Much of this material was originally proposed as a letter to the editor; I have elected to expand it in view of the importance of the subject and the accumulation of new documentary material. This work was performed under the auspices of the U.S. Atomic Energy Commission. I wish to emphasize that I alone am responsible for the opinions herein expressed.
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CURRENT PROBLEMS IN RESEARCH

# Muscle Research

## It is one of the oldest and newest lines of biological inquiry, promising an insight into the nature of life.


Albert Szent-Györgyi


If science is the art of measuring, then muscle has no equal as a material in the study of life, for there is no other tissue whose function is connected with equally extensive and intensive changes in chemistry, physical state, energy, and dimensions. This is why physiology, up to the turn of the century, was mainly muscle physiology. After muscle had been pushed into the background by enzymes and hormones for a while, the development of modern physical methods once more turned attention toward it with its macromolecular organization and its "mechanochemical coupling" (the conversion of chemical bond energy into work).

Muscle also has a strong appeal to the medically minded. The heart and the uterus both are, in a way, but bags of muscle, and our blood pressure is regulated by muscles that determine the lumen of our smaller blood vessels.

The function of muscle is to create motion. There are many sorts of motion, and thus there are many sorts of muscles, even if the basic principles on which they are built may be identical. A muscle cell or fiber is a very complex system, and the unit of its function, the twitch, is a very complex cycle. Hence, "muscle research" covers a wide field of


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inquiry. Fortunately, research is simplified to some extent by the fact that energy production and energy consumption are separated. This separation enables the researcher to work on one of the two processes independently. What is driving the muscle machine is, according to our present knowledge, the free energy released by the splitting of the terminal "high-energy phosphate-bond, ~ P," of adenosine triphosphate (ATP), which is created at the expense of fermentation and oxidation. Oxidative phosphorylation is linked to the mitochondria, while contraction is the function of the contractile filaments.

There are many approaches to muscle. We can inquire, for instance, into the physical changes accompanying contraction, measuring heat production or changes in elastic properties, as A. V. Hill and his associates have done. We can inquire into the nature of the single parts of the contraction cycle, asking how depolarization is produced on the muscle membrane, how this depolarization is propagated, how it triggers the function of the contractile matter inside the fiber, and how the contracted muscle returns to its resting state. We may inquire into the nature of the contractile material and the changes which it undergoes in contraction and subsequent relaxation, and we may inquire into the feedback mechanisms which adjust motion to the physiological requirements. Since each of these partial processes represents a more or less self-consistent field of inquiry, it is impossible to cover all of them within the boundaries of a short article. Accordingly, I shall limit myself to one aspect only, one to which most of my personal experience relates: the problem of the mechanochemical coupling and the nature of the main contractile protein, myosin.

## Early Work on Myosin

Myosin has been known for almost a century, having been discovered by W. Kühne, who showed that a great amount of a protein can be extracted from muscle by a strong salt solution. This protein precipitated on dilution of the salt present and was found in the 1930's by Edsall, Muralt, H. H. Weber, and others to consist of rod-shaped molecules. When I embarked on muscle research two decades ago it became increasingly clear that what was driving contraction was the ~ P of adenosine triphosphate. Engelhardt and Ljubimowa (*1*) had just discovered that myosin could split this bond and thus release the energy which it needed for its contraction. The idea of a "contractile enzyme" was most exciting. None of us had much doubt, then, that contraction had to be some sort of a folding, elicited in the myosin rodlets by the ATP molecule at certain points, and we were looking forward to the possibility of describing this reaction soon by a simple chemical equation.

The only trouble was that myosin would not contract outside the body. My associates, Banga and Straub, and I showed (*2*) that this failure was due to the fact that the contractile protein was not myosin but actomyosin, a complex of myosin with a hitherto unknown protein, "actin." About the same time Schramm and Weber (*3*) showed "myosin" to be dishomogeneous in the ultracentrifuge. Under the electron microscope (Ardenne and Weber, *4*) the faster sedimenting fraction was found to consist of filaments which were, evidently, filaments of actomyosin.

In the resting muscle there seems to be no interaction between actin and myosin, the formation of actomyosin being brought about by "excitation." The association of actin and myosin goes hand in hand with the increase in elastic modulus which characterizes the "active state" of A. V. Hill (*5*). Once it has been formed in the presence of physiological concentrations of ATP and ions, actomyosin has to go over into its contracted state. The energy spent in this process can be used to lift a weight—that is, to do work.

What made actin exciting was the fact that it allowed us to produce and study motion and contraction in vitro, and bolstered our hopes that soon we would know all about the process. If ATP was added to actomyosin in the test tube, the actomyosin underwent violent physical changes which consisted in the shortening of its filaments and the loss of its hydrophilous character. The analogy between these in vitro reactions and muscular contraction could be brought closer by showing that a muscle, thoroughly extracted with glycerol, is still capable of contracting and developing maximal tension on addition of physiological concentrations of ATP (*6*). (Glycerol destroys the finer mechanisms but leaves actomyosin intact.) So the conclusion could be drawn that muscular contraction, essentially, is an interaction of actin, myosin, ATP, and ions. I will omit the discussion of actin and limit myself to myosin.

## Complex Nature of Myosin

The first experimental evidence that the situation was not as simple as we believed and that myosin is not a homogeneous rodlet was obtained by Gergely (*7*) and Perry (*8*), who showed that trypsin decreased the viscosity of myosin solutions without decreasing its ATP-ase activity. The myosin, thus treated, could be separated into two fractions, only one of which showed enzymic activity. After studies pursued with Mihalyi (*9*), the final analysis of this change was given by A. G. Szent-Györgyi (*9*), who showed that the "myosin molecule" is disintegrated by trypsin into six subunits, *meromyosins,* which were shunted in a row, in series. There are two different kinds of such subunits. One kind was thicker and sedimented faster than the other and was, accordingly, called "H" (heavy), while the other was slender and had a lower molecular weight and was called "L" (light). The H meromyosin had the full ATP-ase activity of the whole myosin molecule and interacted with actin, while the L seemed to be involved in shortening. The nature of the links holding the meromyosins together has not yet been cleared up definitely. All the same, these findings made it certain that the myosin particle is not a homogeneous rodlet but consists of different parts with different structures and functions. The L meromyosin has a high, the H a low, α-helix content (Cohen *et al., 10*). That these subunits are, in one way or another, preformed in myosin is also shown by their different amino acid turnover numbers (Velick, *11*).

The situation was somewhat simplified by Laki and Carroll's (*12*) finding that carefully extracted myosin had only half of the previously accepted molecular weight; "old myosin" was thus a dimer formed in vitro after extraction. As far as its dissociating action on actomyosin is concerned, ATP seems to react with myosin in stoichiometric proportions (Hanson and Mommaerts, *13*). To compensate for this simplification, it was found that the meromyosins themselves are built of a great number of much smaller subunits into which they disintegrate if they are acted upon by urea. The L type disintegrates completely, the H partially (A. G. Szent-Györgyi and Borbiro, *14*). The molecular weight of these sub-subunits, "protomyosins," is about 1/100 that of myosin. What is disturbing about this finding is the fact that urea is known to split hydrogen bonds only, leaving covalent bonds intact. If

we define a molecule as a structure with a covalent backbone, then the "myosin molecule" is no molecule at all but a complex system of small units held together by secondary forces, like H-bonds, van der Waals attractions, or dipole moments.

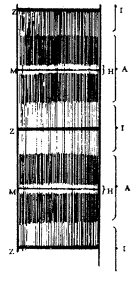
### Function and Chemistry

As a rule, new knowledge leads to a better understanding. With muscle, things seem to go in the opposite direction, and one may ask whether the real difficulty does not lie in an inadequacy of our basic concepts. Present biochemistry stands under the domination of classical chemistry, according to which two molecules must come into bodily contact to be able to interact. This woud mean that the ATP molecules can induce changes in the contractile protein only at the points at which they are bound and split. The fact that only the H meromyosin splits ATP, while it is the L which seems to be more directly involved in contraction, suggests the inadequacy of the classical concept, making some sort of a migration of energy seem likely. This calls to mind the case of the *Bacillus proteus*. This bacterium has long flagellums, about as long as a sarcomere. These flagellums move the bacterium by means of the undulatory motion passing along their whole length. According to their x-ray spectrum, as shown by the studies of Astbury, Beighton, and Weibull (*15*), these very thin, threadlike structures are closely related to myosin and have about the same diameter as the contractile filaments of muscle. Thus, in them we see "biological movement stripped to its barest essentials." Since these flagellums are too thin to allow us to suppose that circulation takes place inside them, the energy which moves them must be fed into them at their basal end and then, somehow, must migrate along their length. Perhaps we have taken a much too narrow view of life in trying to explain all its reactions in terms of classical chemistry. In order to understand we might have to descend from the dimension of macromolecules to those of electrons, from classical chemistry to quantum mechanics, taking into account factors such as molecular excitations, the resonance transfer of their energy, solid-state physics, the electromagnetic field and its perturbations, long-range water structures, and, possibly, proton conduction. Everything seems possible at present. Our knowledge of muscle is in the liquid state.

### Function and Structure

Looking out for some more solid hold, one can try to correlate the known chemical data with the classical microscopic structure of muscle. Such an attempt was made lately by Holtzer and Marshall (*16*), who applied Coons' (*17*) "fluorescent antibody method" to muscle, injecting the various muscle proteins and their subunits into rabbits and then making visible the immune bodies thus produced by coupling them with a fluorescent dye. These workers found that the different immune bodies were bound differently by the different parts of the sarcomere. The "myosin antibody" was bound by the A-band. This finding supported earlier findings (Amberson, *18*; Hasselbach, *19*; Hanson and Huxley, *20*) that myosin is located in the A-band. The "L-antibody" was bound by the lateral parts of the A-band, while the "H-antibody" was bound by the narrow M-band, lying in the middle of the sarcomere, suggesting that this band is its location, and there may be no such thing as myosin in muscle at all. What we called "myosin" might have been an aggregate of meromyosins formed after their extraction.

Another approach was opened by the polarization microscope of Shinya Inoué (*21*). This instrument, with its high resolution and clean polarization optics, reveals new structural details and shows new cross bands. It also indicates that the A-band contains a relatively great quantity of a structural protein which is neither myosin nor actin and which seems to be identical with the "X-protein" (*22*). The microscope also shows that muscle fibrils from which myosin has been extracted bind H meromyosin with preference in the M-band.



The muscles which move our body consist of fibers of the dimension of a human hair. Under the microscope (schematic representations above) these fibers are found to be built of darker, denser, doubly refractant segments (the anisotropic "A-bands"), and lighter, less dense segments with poor double refraction (the isotropic "I-bands"). In the middle of the I-bands are the "Z-membranes." The segments enclosed by two Z-membranes are called "sarcomeres." In the middle of the A-band there is a thin membrane, the "M-membrane," delimited on either side by a narrow zone of small density, the "H-band."

In considering the problem of correlating structure with function and chemistry, one's thoughts naturally turn to the electron microscope, which extended the domain of morphology into macromolecular dimensions. The first attempt on this line is linked to the names of Hall, Jakus, and Schmitt (*23*), who showed that the muscle fiber, essentially, is a bundle of a great number of thin filaments which do not bend or fold in contraction. New details were revealed lately by the admirable pictures of H. Huxley (*24*) which show the presence of two kinds of filaments in cross-striated muscle. There are thicker "primary" filaments, located in the A-band, and twice as many thinner "secondary" filaments reaching from the Z-band to the H-band. In cross sections the thinner filaments were found to surround the thick ones in a hexagonal array.

### Sliding Filaments

On stretching, the two kinds of filaments were found to be sliding past one another, making the H-zone and I-band wider. Building on these observations, Hanson and Huxley (*25*) proposed a new theory of contraction according to which what happens in this process is the opposite of what happens on stretching: the secondary filaments are pulled in between the primary ones with a consecutive gradual narrowing of the I-band, which disappears altogether when the Z-membrane reaches the A-band. A. F. Huxley's (*26*) motion pictures of living muscle strongly plead for this mechanism of contraction, which explains also the puzzling fact that there is no change in x-ray periodicities in initial states of contraction: the muscle shortens but its filaments do not.

No doubt, this theory signifies an important step in the study of muscle. It gives a clear picture of the mechanics and the morphological changes taking place in the contraction of cross-striated muscle, offering a solid foundation for further discussion. But do we really understand muscle now? Far from it; muscle has remained just as much a mystery as it was before. We still do not know what happens when ATP is split and how its energy is, eventually, converted into the pull exerted on the secondary

filaments. The in vitro reaction of actin, myosin, and ATP shows that there are interactions between these substances leading to violent physical changes. Though physical (A. F. Huxley, *27*) and chemical (H. H. Weber, *28*) theories are not lacking, the nature of these interactions is still unknown. They represent the primary happening and form the core of the problem of muscular contraction. Within the framework of the macromolecular arrangements of cross-striated muscle, they cause the secondary filaments to be pulled in between the primary ones, but if this "pulling in" is all there is to it, then shortening should stop at 30 to 40 percent—as soon as the Z-membranes reach the A-band. All the same, muscle can go on shortening up to 80 percent, producing tension all the time. These high degrees of shortening, in cross-striated muscle, may not be physiological, corresponding to the "delta state" of Ramsay, in which changes begin to be irreversible (*5*). All the same, for the theory they are of prime import. Smooth muscles which have no cross bands, and, accordingly, no periodic double array of filaments, also contract up to 80 percent, though they do so at a slower rate. Similarly, actomyosin filaments can contract under the influence of ATP up to 80 percent, though "sliding" makes no sense at all here. So it seems that the sliding of filaments is linked to the specific steric arrangements in cross-striated muscle, where this sliding makes rapid shortening possible, being the secondary consequence of changes which we fail to understand.

## Conclusion

So we can sum up by saying that we still do not understand muscle and do not know how ATP is driving it. It may be true not only that our outlook on biological action is too narrow, but also that our knowledge of muscle structure is too incomplete. Important structures, such as the "endoplasmic reticulum" (Porter and Pallade, *29*), have been discovered lately, and there is no reason to believe that this structure is the last unknown. Important protein fractions (*22*) wait for identification, while other fractions, such as Bailey's tropomyosin (*30*) have not yet been fitted into the muscle machine. The dimensions indicate that the myosin filaments are many molecules thick. So we have to suppose that, just as protomyosins have to join in a very specific way to form a myosin molecule (if there is such a substance at all), so the myosin molecules have to join in a very specific way to build a filament —structural details, without the detailed knowledge of which we can hardly hope to understand function. The painstaking and extensive application of current methods may yield a great deal of important new information, but it is possible that entirely new approaches are needed. Such new approaches are being opened in various quarters. Koshland's (*31*) application of the isotope techniques has already led to surprising new data. The magnetic anisotropy of muscle, discovered recently by Arnold, Mueller, and Steele (*32*) in my laboratory, may lead to new clues.

There is a certain urgency about solving all these riddles, for only a better understanding of muscle can enable us to cope with its disorders, which cause so much suffering. The number of dystrophic patients in this country alone goes into the hundred thousand, and so does the number of lives lost because of hormonal disturbances of the membrane activity of uterus muscle cells (Csapo, *33*). We can hope that a better understanding of muscle will not only spare human suffering and frustration but that it will bring us closer, also, to the understanding of the basic principles on which life is built.

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